

PRE-EMPTION OF HUMAN CELL-MEDIATED LYMPHOLYSIS  
BY A SUPPRESSIVE MECHANISM ACTIVATED IN MIXED  
LYMPHOCYTE CULTURES\*

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The regulation of B-cell and T-cell immune responses has been extensively examined and in the experimental animal appears to involve regulatory or "suppressor" T cells (1-4). The limitations of in vitro experimentation have made comparable study of nonpathological human suppression quite difficult (5). We report here an in vitro method that generates and quantitates suppressor activity in man after antigen-specific activation in mixed leukocyte culture (MLC).

The one-way MLC induces both a proliferative response (6) and the generation of cytotoxic T lymphocytes (CTLs) (7). Both of these responses are mediated by antigen-specific T-cell subpopulations (8, 9) and have been correlated with recognitive and destructive phases of allograft rejection. Recent reports have examined the antigen reactivity of mouse (10, 11), rat (12), or human (13, 14) lymphocytes obtained after proliferation in MLC. In all cases, after the primary MLC proliferative peak, the recovered lymphocytes rapidly differentiate upon re-exposure to the initial stimulating population, but do so only weakly when exposed to a presumably noncross-reactive third-party stimulating population.

Velocity sedimentation separation studies have shown that the blast cells produced in a primary MLC revert to small lymphocytes that rapidly differentiate into proliferating and/or cytotoxic T lymphocytes upon restimulation with the initial antigen (15). These findings demonstrate that positive selection for the responding population in primary MLC does exist and may account for at least part of the specificity of the secondary response. However, this positive selection does not preclude possible involvement of a suppressor mechanism. In fact we have detected suppressor activity in primary MLC sensitization cultures at a time when the proliferation responsible for positive selection is not yet significant, suggesting that suppression may be of overriding importance in the specificity of MLC-activated secondary responses.

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TABLE I  
*Reciprocal CML Pre-emption*

CTLs			Cytotoxicity							
		Day 6				Day 8				
Day 0	Day 2	B targets K/T		C targets K/T		B targets K/T		C targets K/T		
		30	6	30	6	30	6	30	6	
		%		%		%		%		
1	AB <sub>m</sub>	—	54	33	14	2	33	12	5	3
2	AC <sub>m</sub>	—	8	2	60	37	1	1	46	28
3	AB <sub>m</sub> C <sub>m</sub>	—	44	30	61	35	34	14	52	24
4	AB <sub>m</sub>	C <sub>m</sub>	50	38	29	18	43	22	11	6
5	AC <sub>m</sub>	B <sub>m</sub>	21	14	69	48	12	2	64	35
6	A	C <sub>m</sub>	6	4	28	16	9	2	65	45
7	A	B <sub>m</sub>	21	15	5	3	48	24	10	3
8	AA <sub>m</sub>	C <sub>m</sub>	—	—	—	—	7	2	70	44
9	AA <sub>m</sub>	B <sub>m</sub>	—	—	—	—	53	28	7	3
10	BC <sub>m</sub>	—	1	1	57	30	—	—	—	—
11	CB <sub>m</sub>	—	40	24	-4	-1	—	—	—	—

Each culture flask contained  $9 \times 10^6$  responding cells and  $12 \times 10^6$  stimulating cells added on day 0 or day 2. If two populations of stimulating cells were added,  $6 \times 10^6$  of each were used. All CML combinations were tested on days 6 and 8 at killer to target ratios (K/T) of 30 and 6 to 1. Control value counts per minute for each target: Day 6 B Spontaneous Release (SR) = 156 Max = 1,079; Day 6 C SR = 198, Max = 852; Day 8 B SR = 168, Max = 1,498; Day 8 C SR = 163, Max = 1,105.

## Materials and Methods

Methods used for generating CTLs and performing cell-mediated lympholysis (CML) assays in these studies have recently been described (16). All lymphocytes were Ficoll-Hypaque purified and obtained from healthy nontransfused volunteers. The protocols involved in these suppression studies required CML testing on two target populations that were minimally cross-reactive in the CML assay; however, this, relatively sensitive CML assay detects extensive cross-reactivity between most individuals examined. Therefore, many individuals were screened by prior CML testing to identify five different groups of three donors each. To simplify presentation of data, the responding cell donor in each group is designated A, and the other two B and C. These three individuals were in each case chosen so that CTLs from an AB<sub>m</sub> culture induced strong specific killing on B targets and low cross-killing on C targets; the CTLs from an AC<sub>m</sub> culture killed reciprocally.

## Results

When fresh lymphocytes from individual A are simultaneously sensitized with mitomycin C-treated cells from B and C (B<sub>m</sub> and C<sub>m</sub>), cytotoxicity is developed against both B and C targets. CML blocking studies have shown that such cultures generate separate populations of CTLs, one reactive to antigens on B and the other reactive to C (16). Unlike simultaneous addition of B<sub>m</sub> and C<sub>m</sub>, the addition of C<sub>m</sub> to an ongoing AB<sub>m</sub> culture that was initiated 2 days earlier induces very little cytotoxicity on C, but allows the development of normal cytotoxicity on B targets. In this case the ongoing AB<sub>m</sub> culture "pre-empts" the cytotoxic response of the subpopulation of A cells able to recognize C.

An example of CML pre-emption is presented in Table I. In the standard day 6 CML assay, individual A demonstrated great specificity in distinguishing B target cells from C target cells (rows 1 and 2); however, even this low level of cross-killing was greater than the "autokilling" (rows 10 and 11). Rows 3 and 4 represent CTLs obtained from sensitization flasks that contained the same

number of A, B<sub>m</sub>, and C<sub>m</sub> cells, the only difference being that fresh C<sub>m</sub> cells were added on day 2 in row 4, as opposed to on day 0 in row 3. These two populations killed B target cells to the same extent, as did the CTLs sensitized to B<sub>m</sub> alone (row 1). Cytotoxicity on C targets by the AB<sub>m</sub>C<sub>m</sub> culture (row 3) was similar to that mediated by the AC<sub>m</sub> culture (row 2); both of these were much greater than that mediated by the AB<sub>m</sub> culture stimulated on day 2 with C<sub>m</sub> (AB<sub>m</sub>-C<sub>m</sub>, row 4). The cytotoxicity mediated on C targets by  $30 \times 10^4$  CTLs from this pre-empted culture (row 4) was 29%, less than that mediated by only  $6 \times 10^4$  CTLs in rows 2 and 3. Because peak CML activity is observed 6 days after allogeneic stimulation, it is essential to examine the cytotoxic activity of these cultures on day 8, the expected time of peak response to the stimulating cells added on day 2. Of greatest importance was the cytotoxicity mediated by the "pre-empted" mixture (row 4). Like rows 1 and 3, cytotoxicity on B targets dropped from day 6 to day 8. However, unlike the increased cytotoxicity directed at C targets in rows 6 and 8, the day 8 cytotoxicity on C targets by this pre-empted combination was as low as the cross-killing by AB<sub>m</sub> on the C targets (row 7). This indicated that the response of the A lymphocytes to the addition of C<sub>m</sub> cells on day 2 was pre-empted by the ongoing response to B<sub>m</sub>-stimulating cells. That the C<sub>m</sub> cells obtained on day 2 were highly stimulatory was demonstrated by the cytotoxicity on C target cells in rows 6 and 8; the percent cytotoxicity was of comparable magnitude to that observed on day 6 using the standard CTLs stimulated on day 0 with C<sub>m</sub> cells (rows 2 and 3).

The reciprocal combination and its appropriate controls also demonstrated that the ongoing response to C<sub>m</sub> pre-empted the generation of CTLs to B target cells when B<sub>m</sub>-stimulating cells were added on day 2 (row 5). This pre-emption is similar to preliminary data obtained in mouse (17).

In other experiments, pre-emption in man was observed by adding C<sub>m</sub> to a 24-h ongoing AB<sub>m</sub> culture; the pre-emption effect increased slightly from day 1 to 3 (80–96% inhibition of cytotoxic potency). The observation of pre-emption before detectable MLC or CML reactivity and 4–5 days before their peaks would not be expected from a mechanism involving only positive selection for the responding (proliferating) population.

Direct evidence supporting a suppressive mechanism is presented in Table II. Fresh C<sub>m</sub>-stimulating cells were added to ongoing 2 day AB<sub>m</sub> or AA<sub>m</sub> cultures with or without fresh A lymphocytes. The AB<sub>m</sub> cultures pre-empted the cytotoxic response to fresh C<sub>m</sub> alone (row 5 compared to rows 4 and 6). If this pre-emption were merely selective in nature it would not be expected to influence fresh A lymphocytes from responding to C<sub>m</sub>. However, the ongoing AB<sub>m</sub> culture (row 8), but not the AA<sub>m</sub> culture (row 7), markedly suppressed the expected development of cytotoxicity directed towards C (row 3) when fresh A plus C<sub>m</sub> cells were added to them on day 2.

The combinations presented in the last four rows involved the addition of fresh A cells on day 2 and fresh C<sub>m</sub> cells on day 3. Again, the ongoing AB<sub>m</sub> response initiated on day 0 suppressed the generation of CTLs directed against C. The observed suppressive effect required the responding AB<sub>m</sub> cells, since the cell-free supernate from a 2 day AB<sub>m</sub> culture did not suppress (row 12).

In other experiments, cells from a AB<sub>m</sub> sensitization flask were removed and

TABLE II  
*In Vitro Induced CML Suppression*

	CTLs			Day 8 cytotoxicity			
				B targets		C targets	
	Day 0	Day 2	Day 3	K/T ratio		K/T ratio	
				25/1	5/1	25/1	5/1
						%	
1	AB <sub>m</sub>	—	—	58	33	5	-1
2	AC <sub>m</sub>	—	—	5	1	60	38
3	—	AC <sub>m</sub>	—	5	3	73	39
4	AB <sub>m</sub> C <sub>m</sub>	—	—	51	27	62	33
5	AB <sub>m</sub>	C <sub>m</sub>	—	64	29	15	6
6	AA <sub>m</sub>	C <sub>m</sub>	—	1	1	52	32
7	AA <sub>m</sub>	AC <sub>m</sub>	—	2	0	64	34
8	AB <sub>m</sub>	AC <sub>m</sub>	—	74	42	32	15
9	AA <sub>m</sub>	A	C <sub>m</sub>	4	5	48	32
10	AB <sub>m</sub>	A	C <sub>m</sub>	68	43	20	8
11	—	A + med*	C <sub>m</sub>	0	0	47	23
12	—	A + sup‡	C <sub>m</sub>	0	1	46	27

CML reactions were performed with CTLs obtained from sensitization flasks to which responding and stimulating cells were added on days 0, 2, or 3. Each flask received a total of  $9 \times 10^6$  responding cells and  $12 \times 10^6$  stimulating cells. Control values for each target: B targets: SR = 179, Max = 1,441; C targets: SR = 379, Max = 1,904.

\* Fresh A lymphocytes were cultured on day 2 in medium that had been cultured since day 0 in the absence of cells.

‡ Fresh A lymphocytes were cultured on day 2 in cell-free supernate from AB<sub>m</sub> culture established on day 0.

washed after 2 days of culture and added to fresh A plus C<sub>m</sub> cells; suppression similar to the above was caused by these cells, while the 2 day AB<sub>m</sub> supernate had no suppressing effect.

To determine where the suppressive mechanism was acting, cells from an ongoing 2-day AB<sub>m</sub> culture were added to an ongoing 2-day AC<sub>m</sub> culture. The cytotoxicity observed on day 6 was similar to that from a simultaneously stimulated AB<sub>m</sub>C<sub>m</sub> culture, showing no suppressive effect. This suggests that the suppression mechanism demonstrated above involves inhibition of immune recognition or of the early steps in CTL differentiation.

### Discussion

These experiments have demonstrated that a cell-dependent suppression of CTL activation is generated in human MLC. Several distinct methods of generating and detecting suppressor activity have recently been described (1-5, 18-22); aspects of this *in vitro* suppression in man appear to parallel certain qualities of *in vitro* induced murine suppression (23). However, more studies are required to determine the *in vivo* significance and the specific cellular mechanism of this suppression. Many complex models could be constructed to account for the phenomena, yet this seems unwarranted until more insight is provided. At present, two conclusions can be derived from these studies. Firstly, cell-mediated suppression of immune responses can be generated and studied *in vitro* using human lymphocytes responding to allogeneic cells. Secondly, the specificity of secondary responses to alloantigens after sensitization in MLC represents,

at least in part, a "pre-emption" of third-party responsiveness by this suppression mechanism.

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